p-Anilinoaniline Enhancement of Dioxin-induced CYPIA1 Transcription and Aryl Hydrocarbon Receptor Occupancy of CYPIA1 Promoter: Role of the Cell Cycle

Althea Elliott, Aby Joiakim, Patricia A. Mathieu, Zofia Duniec-Dmuchowski, Thomas A. Kocarek and John J. Reiners, Jr.

Institute of Environmental Health Sciences
Wayne State University
Detroit, MI 48201
Abstract

The aryl hydrocarbon receptor (AhR) is targeted by ubiquitination for degradation by the proteasome shortly after its activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In silico screening identified p-anilinoaniline (pAA) as a putative inhibitor of an E2 ligase that partners with an E3 ligase implicated in AhR ubiquitination. We investigated if pAA could modify AhR-dependent activation of its target gene CYP1A1. pAA (1-200 μM) alone did not affect AhR content, or stimulate CYP1A1 mRNA accumulation in human mammary epithelial MCF10A cultures. However, pretreatment with ≥ 100 μM pAA suppressed TCDD-induced CYP1A1 activation and AhR degradation via its functioning as an AhR antagonist. At a lower concentration (25 μM) pAA cotreatment increased TCDD-induced CYP1A1 mRNA accumulation, without inhibiting AhR turnover, or altering CYP1A1 mRNA half life. Whereas TCDD alone did not affect MCF10A proliferation, 25 μM pAA was cytostatic, and induced a G1 arrest lasting ~7 h, and a S phase arrest that peaked 5-8 h later. TCDD neither affected MCF10A cell cycle progression, nor did it alter pAA effects on the cell cycle. The magnitude of CYP1A1 activation depended upon the time elapsed between pAA pretreatment and TCDD addition. Maximum AhR occupancy of the CYP1A1 promoter, and accumulation of CYP1A1 hnRNA and mRNA occurred when pAA pretreated cultures were exposed to TCDD in late G1 and early/mid S phase. TCDD-mediated induction of CYP2S1 was also cell cycle dependent in MCF10A cultures. Similar studies with HepG2 cultures indicated that the cell cycle dependency of CYP1A1 induction is cell context dependent.
Introduction

In many cell types the AhR undergoes proteolysis following the binding of agonist. For example, TCDD binding reduces AhR half-life from 28 h to ~3 h in murine hepatoma 1c1c7 cells (Ma and Baldwin, 2002). AhR degradation occurs as a consequence of its becoming poly-ubiquitinated, which targets it to the proteasome for proteolysis (Pollenz, 2002). Poly-ubiquitination is mediated by the sequential actions of three interacting, but functionally distinct proteins: an E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzymes and the E3 ubiquitin ligases (Pickart, 2001). There are about 50 known E2s and possibly hundreds of E3 proteins. The E3 proteins are responsible for substrate discrimination and the specificity of ubiquitination. A single E2 is able to associate with different E3s, and an individual E3 may associate with more than one E2 (Hershko and Ciechanover, 1998). Although the identities of the E2 and E3 proteins involved in ligand-induced AhR ubiquitination are not well characterized, some studies have implicated a role for the E3 ligase CHIP (Lees et al., 2003; Morales and Perdew, 2007). CHIP contains a U-box domain, and interacts with hsp90 and hsc70 client proteins targeting them for degradation by the ubiquitin proteasome pathway (Connell et al., 2001). One of the E2 conjugating enzymes that partners with CHIP is UbcH5a (Jiang et al., 2001).

\( p \)-Anilinoaniline (\( p \)AA; also known as \( p \)-aminodiphenylamine, N-phenyl-1,4-benzenediamine) is an aromatic amine that is used in hair coloring products. It is also a major metabolite of the azo dye metanil yellow (Srivastava et al., 1982). The latter is extensively used in the textile, paper, lacquer/stain industries (Mittal et al., 2008), and in India as a food-coloring agent (Khanna et al., 1985). As the consequence of in silico screening, Banerjee (2006) identified
pAA (identified as 05RB in the paper) as a putative inhibitor of UbcH5a, an E2 conjugating enzyme that partners with the E3 ligase CHIP.

We initiated the current study with the intent of assessing pAA as a possible inhibitor of TCDD-induced AhR ubiquitination in the normal human mammary epithelial cell line MCF10A. We previously reported that TCDD induced AhR degradation and the expression of multiple AhR-responsive phase I and II metabolism-related genes (i.e., \textit{CYP1A1}, \textit{CYP1A2}, \textit{CYP1B1} and \textit{NQO1}) in MCF10A cultures (Reiners et al., 1997; Gou et al., 2004; Jojakim et al., 2004). Unfortunately, MCF10A cultures ultimately proved to be an inappropriate model for the testing of our hypothesis since the line did not express UbcH5a. However, we found that pAA mediated a very reproducible time-dependent enhancement of \textit{CYP1A1} induction by TCDD that paralleled pAA-induced cell cycle arrest and release. Specifically, our data indicate that TCDD-induced AhR occupancy of the \textit{CYP1A1} promoter and \textit{CYP1A1} transcriptional activation in MCF10A cells are maximal in late G\textsubscript{1} and early/mid S phase cells.
Materials and Methods

Materials. TCDD was purchased from Midwest Research Institute (Kansas City, MO). TRIzol, trypsin/EDTA, epidermal growth factor, penicillin/streptomycin solution, horse serum, salmon sperm DNA, Taq DNA polymerase, phenol, Random Primers DNA Labeling System, PCRx Enhancer System, and PCRx Amplification mixture were purchased from Invitrogen (Carlsbad, CA). p-Anilinoaniline (pAA), actinomycin D, dimethylsulfoxide (DMSO), deoxyribonucleotide (dNTP) mix, deoxyribonuclease 1, protease inhibitor cocktail, protein A agarose, and ribonuclease A were obtained from Sigma-Aldrich (Milwaukee, WI). RNAqueous-4PCR kit was obtained from Ambion Inc. (Austin, TX). TaqMan Reverse Transcription Reagent, TaqMan Gene Expression Assays and SyberGreen PCR master mix were from Applied Biosystems (Foster City, CA).

Cell culture. MCF10A human breast epithelial cells were obtained from the Cell Lines Resource, Karmanos Cancer Institute (Detroit, MI) and maintained as attached cultures in supplemented Dulbecco’s Modified Eagle medium/Ham’s F-12 medium as described by Guo et al. (2001). HepG2 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco’s Modified Eagle Medium supplemented with non-essential amino acids, 100 units/ml penicillin, 100 mg/ml streptomycin (Gibco-BRL, Carlsbad, CA) and 10% fetal bovine serum (Fisher Scientific, Hanover park, IL). Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂. Cells were seeded at densities that assured exponential growth for at least 5 days. Cultures were treated on the second day of plating with either DMSO or different concentrations of pAA dissolved in DMSO. Solvent never exceeded 0.1%. For estimates of cell number and viability cultures were trypsinized, washed
with PBS, suspended in PBS containing trypan blue, and counted with a haemocytometer. Viability was scored as the ability to exclude trypan blue.

**Cell Cycle Analyses.** The procedure used for the determination of cell cycle phase by fluorescence activated cell sorting has been described in detail (Reiners et al., 1999).

**Western Blot Analyses.** The conditions used for the preparation of cell extracts, separation of proteins on SDS-polyacrylamide gels, and transfer of separated proteins onto nitrocellulose membranes have been described in detail (Guo et al., 2001). Nonspecific antibody binding to transferred proteins was blocked by pre-incubating membranes in PBS-T (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20) supplemented with 5% non-fat dry milk proteins. After washing with PBS-T, membranes were incubated with the appropriate horseradish peroxidase secondary antibody for 1.5 h at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction kit (Amersham Pharmacia Biotech, Piscataway, NJ), and recorded on x-ray film.

**RNA Preparation and Northern Blot Analyses.** The conditions used for RNA isolation, resolution of RNAs on agarose/formaldehyde gels, and transfer to nitrocellulose membranes have been described by Reiners et al. (1997). The probes and hybridization conditions used for the detection of human 7S, CYP1A1, and CYP1A2 RNAs have been described in detail (Reiners et al., 1997; Joiakim et al., 2004). Northern blot data were normalized by calculating CYP1A1 or CYP1A2 mRNA to 7S RNA signal strength ratios.

**Real Time RT-PCR of CYP1A1 hnRNA.** Total RNA was isolated from MCF10A cultures using the RNAquous-4PCR kit according to manufacturer’s specifications. RNA was treated with DNase 1 according to the instructions provided by kit’s manufacturer in order to remove trace amounts of DNA, and was quantified using a NanoDrop Spectrophotometer (NanoDrop...
Technologies, Wilmington DE). cDNA was synthesized from isolated RNA using TaqMan Reverse Transcription Reagents, as described by the manufacturer. For quantification of CYP1A1 hnRNA PCR amplification used the CYP1A1 forward primer 5’-TTGTGATCCCCAGGCTCAAGA-3’ and the reverse primer 5’-GGAGGGACCAAAATGTTCCTTT-3’. These primers amplify a region 10569 to 10688 bp downstream of the CYP1A1 transcription start site, and correspond to a region spanning the first exon-intron (Genbank AF253322). For GAPDH mRNA quantification we used the GAPDH forward PCR primer 5’-AGAAAAACCTGCAATATGATGAC-3’ and the reverse PCR primer 5’-GCCCAGGATGCCCCTGA-3’. This amplifies a region 849-927 bp downstream of the transcription start site. PCR data were analyzed using the comparative cycle threshold (Ct) method. Relative quantification was based on relative expression of CYP1A1 normalized against GAPDH. Relative expression of the target gene was calculated as $2^{-\Delta\Delta C_t}$ where $\Delta C_t$ was obtained by subtracting $\Delta C_t$ of untreated cells from the $\Delta C_t$ of treated cells. The $\Delta C_t$ was calculated by subtracting the average Ct values for GAPDH from the average Ct value of CYP1A1. All PCR reactions were performed in triplicate (technical replicates).

**Real Time RT-PCR of CYP1A1 and CYP2S1 mRNAs.** Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), and reverse transcribed using the Omniscript RT kit (Qiagen) and random primers (Invitrogen, Carlsbad, CA) according to the manufacturers’ instructions. Human CYP1A1 and CYP2S1 mRNA contents were quantified as previously described (Duniec-Dmuchowski, et al., 2007), using TaqMan Gene Expression Assays Hs00153120_m1 and Hs00258076_m1, respectively, in multiplex reactions with TaqMan 18S rRNA Endogenous Control (primer limited) to quantify endogenous 18S rRNA (Applied
Biosystems, Foster City, CA). Cycle threshold values were used to normalize CYP1A1 or CYP2S1 levels to 18S RNA levels as described above.

**Chromatin Immunoprecipitation Assay.** Culture medium was adjusted to 1% formaldehyde and incubated for 10 min at room temperature in order to cross-link protein-DNA complexes. Cultures were subsequently rinsed with cold PBS and incubated for 5 min at room temperature with a 125 mM glycine/PBS solution. Cells were then washed with PBS, covered with PBS supplemented with 1x (v/v) protease inhibitor cocktail (Sigma), and detached by mechanical scraping. The cell suspensions were pelleted by centrifugation, quick frozen, and stored at -80°C until further processing. The cell pellets were subsequently mixed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and sonicated using conditions that yielded DNA lengths of 200-1000 bp. The sonicated chromatin was diluted 1:10 with ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.0] and 167 mM NaCl) supplemented with protease inhibitors. Chromatin was pre-cleared with salmon sperm DNA/protein A agarose (60 μl of a 50% slurry), 5 μg of salmon sperm DNA and 1 μg rabbit IgG with gentle agitation for 2 h at 4°C. Agarose beads were pelleted by centrifugation at 700g for 1 min. The supernatant fluid was transferred to a new tube and a sample was put aside for determination of input DNA. One μg of either AhR antibody or rabbit IgG was added to the remaining supernatant fluid. After overnight incubation at 4°C on a rotating platform, salmon sperm DNA/protein A agarose (40 μl of a 50% slurry) was added, and an additional incubation was carried out for 1.5 h at 4°C prior to pelleting the agarose beads by centrifugation. The agarose beads were sequentially washed for 10 min each in 1 ml of low salt wash buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), 1 ml of high salt wash buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1%
SDS), 1 ml of LiCl wash buffer (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 1 mM EDTA, 1% IGEPAL, 1% sodium deoxycholate), and 2 x 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)]. Protein-DNA complexes were eluted from the beads by incubation with 250 μl of 0.1 M NaHCO₃, and 1% SDS for 30 min. Cross-linking was reversed by incubating the supernatant overnight at 65°C in 200 mM NaCl. DNA was subsequently purified by standard procedures.

AhR-DNA interactions were monitored using forward (5’- ACCCGCCACCCTCGACAGTTC-3’) and reverse (5’-TGCCCAGGCGTTGCGTGAGAAG-3’) primers to amplify a region from -980 to -1125 of the CYP1A1 promoter (Matthews et al., 2005). This region contains two functional XREs and yields a PCR product of 146 bp. The PCR reaction mixture contained input or ChIP-isolated DNA, 1x PCRx amplification buffer, 1x PCRx enhancer solution, 0.2 mM of dNTP mix, 2.5 mM MgSO₄, 0.4 μM each of forward and reverse primers, and 0.5 U of Taq DNA polymerase. The PCR reaction employed the following conditions and cycles: 95°C for 3 min, 35 cycles of 95°C for 45s, 58°C for 45s and 70°C for 1 min, and then 70°C for 5 min. PCR products were separated on agarose gels and stained with ethidium bromide.

**Electrophoretic Mobility Shift Assay.** The conditions used for TCDD-mediated AhR transformation in rat liver extracts, EMSA, and the sequence of the double stranded radiolabeled oligonucleotide containing a consensus XRE, have been published (Joiakim, et al., 2004).

**Image and statistical Analyses.** EMSA, western and northern blot films were scanned at 800-1000 dpi and saved as tif images. Band intensities in the images were quantified with ImageJ software. Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test, using GraphPad Prism Version 4.02 for Windows (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant.
Results

Effects of pAA on TCDD induction of CYP1A1. Exposure of MCF10A cultures to 10 nM TCDD induced the accumulation of CYP1A1 mRNA; whereas, exposure to 1 to 200 μM pAA had no effect (Fig. 1A). However, depending upon the concentration employed, pAA pretreatment could either suppress or enhance dioxin-induced CYP1A1 mRNA accumulation (Figs. 1A and B). Specifically, pretreatment with concentrations of pAA ≥ 75 μM suppressed TCDD-induced CYP1A1 mRNA accumulation; whereas, concentrations of pAA ≥ 10 μM and ≤ 50 μM enhanced CYP1A1 mRNA accumulation. Kinetic analyses indicated that induced steady state CYP1A1 mRNA levels were fairly constant following 5 to 18 h of TCDD treatment (Fig. 1C). In contrast, TCDD-induced CYP1A1 mRNA accumulation was notably bell shaped in pAA-pretreated cultures, with maximal accumulation (e.g., ~3 to 4-fold greater than dioxin alone) occurring 7.5 – 12 h after TCDD addition (Fig. 1 C).

CYP1A2, like CYP1A1, is transcriptionally activated by TCDD in MCF10A cultures (Joiakim et al., 2004). pAA alone had no effect on CYP1A2 mRNA content in MCF10A cultures (Joiakim, unpublished data). However, pretreatment with 25 μM pAA enhanced dioxin-induced CYP1A2 mRNA contents by ~2-fold (Fig. 1D). Interestingly, maximal CYP1A1 and CYP1A2 mRNA accumulations occurred within a similar time period (compare Figs. 1C and D).

The accumulation of CYP1A1 mRNA following TCDD treatment could reflect either enhanced CYP1A1 transcription, or a stabilization of CYP1A1 mRNA. In order to examine the former, we made cDNA to total RNA and used PCR to amplify hnRNA sequences that encompassed the exon 1 – intron 1 boundary of CYP1A1 (Fig. 2A). Analysis of hnRNA has been used as a surrogate assay for monitoring the transcriptional activation of CYP1A1 (Elferink and Reiners, 1996). Exposure to either DMSO or 25 mM pAA did not alter CYP1A1 hnRNA content.
over a 8 h time period; whereas, TCDD treatment resulted in an ~20-fold increase (Fig. 2B). Pretreatment with 25 mM pAA, followed by TCDD addition, increased CYP1A1 hnRNA content ~2-fold above what accumulated following 6 h of just TCDD exposure (Fig. 2C).

In order to assess the effects of pAA on CYP1A1 mRNA stability we pretreated cultures with TCDD ± pAA for 8 h before adding 5 μg/ml actinomycin D (ActD), a concentration that completely inhibits CYP1A1 transcription (Chen et al., 1995; Ciolino et al., 1999). Total RNA was subsequently collected at 2 h intervals for measurement of CYP1A1 mRNA (Fig. 3A). Decreases in mRNA content following ActD treatment became very apparent with passing time. Fig. 3B depicts CYP1A1 mRNA half-life analyses based upon data generated in 4 independent experiments. pAA pretreatment had no statistically significant effect on the rate of CYP1A1 mRNA turnover (Fig. 3B).

**pAA modulation of TCDD-induced AhR degradation.** A substantial reduction in AhR content occurred in MCF10A cultures within 6 h of dioxin addition (Fig. 4A). Pretreatment with pAA suppressed TCDD-induced AhR turnover in a concentration-dependent manner (Fig. 4A). A strong inhibition of TCDD-induced AhR loss was observed in cultures cotreated with 100 μM pAA (Fig. 4A, p<0.05 for n = 4 independent experiments), whereas, 10 μM pAA was without effect (Fig. 4A). Kinetic analyses of intermediate concentrations suggested that 50 μM pAA partially blocked TCDD-induced AhR degradation (Fig. 4B, p<0.05 for n = 3 independent experiments). Although 25 μM pAA appeared to exhibit a small protective trend, the AhR contents of TCDD and TCDD + pAA treated cultures were not significantly different (Fig. 4C).

Although the effect of pretreatment with 25-50 μM pAA on AhR content was small, we wondered if it might be sufficient to mediate the enhanced accumulation of CYP1A1 mRNA after TCDD exposure. As an approach to the issue we intended to knock down the putative E2
target of pAA (i.e., UbcH5a). Surprisingly, although we could easily detect UbcH5a in HEK293 cells by western blotting, we were unable to detect it in MCF10A cultures (data not presented). Hence, it seemed unlikely that the effects of pAA on AhR content and CYP1A1 induction were related to the pAA effects on the E2 UbcH5a.

AhR agonist and antagonist activities of pAA. The ability of pAA to suppress both TCDD-induced AhR proteolysis and CYP1A1 transcription at 100 μM is consistent with it functioning as an AhR antagonist. We used an electrophoretic mobility shift assay (EMSA) to examine this issue. Incubation of rat liver cytosol with 10 nM TCDD effectively transformed the AhR into a species capable of binding to a radiolabeled oligo containing a xenobiotic-responsive element (Fig. 5A). In contrast, AhR transformation did not occur following incubation of cytosol with 1-200 μM pAA (Fig. 5A). However, cotreatment of rat liver cytosol with pAA suppressed the formation of TCDD-induced AhR-DNA complexes, in a concentration-dependent fashion (Fig. 5B). This suppression was observed at concentrations of pAA ≥ 50 μM. Hence, the EMSA assay suggests that pAA has no AhR agonist activity, but can function as an AhR antagonist once a critical concentration is reached.

pAA effects on CYP1A1 and CYP2S1 induction are cell cycle dependent. We previously reported that pAA is cytostatic to MCF10A cultures in a concentration-dependent fashion over the range 10 to 50 μM, causing an almost complete suppression of proliferation at 50 μM (Elliott and Reiners, 2008). Cell cycle analyses indicated that 25 μM pAA induced simultaneous G1 and S phase blocks in MCF10A cultures (Fig. 6A). During the first 7 h of pAA treatment the proportion of cells in S phase held fairly constant, while the proportion of G1 cells increased due to the progression of G2/M cells. Thereafter, G1 cells began to transition into S phase where they accumulated for the next 8 h. Arrested S phase cells began to transition into G2/M ~15 h after
pAA addition (Fig. 6A). Notably, the onset of G1 cells transitioning into S phase, and the period of S phase cell accumulation, roughly correlate with when pAA cotreatment enhanced TCDD activation of CYP1A1 (compare Fig. 1C and 6A). This temporal relationship is significant since we previously reported that CYP1A1 induction may be cell cycle regulated (Santini et al., 2001).

In order to examine the relationship between AhR function and cell cycle phase in detail, we quantified TCDD-mediated transcriptional activation of CYP1A1 at different times after pAA treatment. Prior to initiating this study we determined if TCDD had any effects on MCF10A cell cycle progression. A concentration of TCDD sufficient to activate CYP1A1 transcription neither altered cell cycle phase distributions over a 24 h treatment period when used singularly (Supplemental Figure 1), nor altered the development and resolution of pAA-induced G1 and S phase arrest. The latter studies were performed in two ways. In one protocol TCDD was added 1.5 h after pAA addition, and cell cycle analyses were performed throughout a 24 h period (Supplemental Figure 1). In the second protocol we treated cultures with TCDD at various times after pAA addition, and harvested cultures 3 h after dioxin addition (Fig. 6B). This latter protocol facilitated very detailed analyses of TCDD effects on specific phases of the cell cycle. Overall, TCDD had no detectable effects on MCF10A cell cycle progression.

Cultures treated similarly to those in Figure 6B were analyzed for CYP1A1 mRNA (Fig. 6C) and hnRNA (Fig. 6D) following the TCDD addition. In these studies we harvested cultures within 3 h of TCDD addition in order to restrict the effects of dioxin to defined stages of the cell cycle. The length of time elapsed between pAA and dioxin additions influenced the extent to which CYP1A1 mRNA and hnRNA accumulated. CYP1A1 mRNA contents were maximally increased when dioxin was added to cultures in late G1 or transitioning from G1 to S (i.e., time of harvest = 13 h), and in early – middle S phase (i.e., time of harvest = 15 h; Figure 6C). Late S
phase cells (i.e., time of harvest = 18h) exhibited less CYP1A1 mRNA, and CYP1A1 mRNA content continued to drop as S phase cells transitioned into G2/M (Fig. 6C). The kinetics of CYP1A1 hnRNA accumulation and decline paralleled CYP1A1 mRNA content (compare Figs. 6C and D). Chromatin immunoprecipitation (ChIP) assays with AhR antibodies did not detect AhR occupancy of the CYP1A1 promoter in non-treated cultures, or cultures treated with only pAA for 1.5, 11 or 18 h (Fig. 6E). In pAA pretreated cultures, AhR occupancy of the CYP1A1 promoter following TCDD addition qualitatively correlated with CYP1A1 hnRNA levels (compare Figs. 6D and E). Specifically, AhR occupancy of the CYP1A1 promoter was markedly less in cultures treated with TCDD 1.5 h after pAA addition, relative to cultures treated with TCDD 11 and 18 h after pAA addition.

CYP2S1 is transcriptionally activated by TCDD through an AhR-dependent mechanism (Saarikoski et al., 2005; Riveria et al., 2007). Thomas et al. (2007) previously reported that CYP2S1 mRNA accumulates in MCF10A cultures following the addition of dioxin. In asynchronous MCF10A cultures we observed no accumulation of CYP2S1 mRNA within 3 h of TCDD addition (Fig. 7A). However, this time period was sufficient for CYP2S1 mRNA accumulation if the cultures were pretreated with pAA (Fig. 7A). Like CYP1A1, optimal accumulations of CYP2S1 mRNA occurred when cultures were treated with TCDD in late G1, during the G1/S transition and early/mid S phase (Compare Figs. 7A and B, hours 5-12 after pAA addition).

pAA effects on CYP1A1 induction in HepG2 cultures. We examined the effects of pAA in HepG2 cells in order to determine if its effects on CYP1A1 induction were cell type specific. pAA suppressed the proliferation of HepG2 in a concentration-dependent fashion (Fig. 8A) that mirrored the effects observed in MCF10A cells (Elliott and Reiners, 2008). Concentrations of
$pAA \geq 25$ $\mu M$ were very cytostatic, with antiproliferative effects that persisted for at least 72 h. The cytostatic effects of $25 \mu M$ $pAA$ reflected the induction of an early $G_1$ and $S$ phase arrest, with arrested $G_1$ phase cells transitioning into $S$ phase 8-16 h after $pAA$ addition (Fig. 8B). Unlike MCF10A cultures, $pAA$-treated cultures remained arrested in $S$ phase 28 h after treatment (Fig. 8B). Treatment of asynchronous HepG2 cultures with TCDD increased $CYP1A1$ mRNA content 11- to 19-fold within 3 h of dioxin addition (Fig. 8C). Pretreatment with $pAA$ increased TCDD-induced $CYP1A1$ mRNA accumulation above that achieved with just TCDD (Fig. 8C). However, unlike what we observed with MCF10A cultures, the effects of $pAA$ pretreatment on TCDD-mediated $CYP1A1$ induction were not cell cycle dependent. Instead, $pAA$ enhanced $CYP1A1$ mRNA accumulation irrespective of the cell cycle stage at which TCDD was added (Fig. 8C).

Although $CYP2S1$ mRNA was detected in the non-treated asynchronous HepG2 cultures reported in Fig. 8, we observed no induction of $CYP2S1$ in these cultures following a 3 h treatment with TCDD, or combined $pAA$ and TCDD cotreatment (unpublished data).
Discussion

We initiated the current study in order to determine if pAA was a modulator of agonist-activated AhR degradation. Unexpectedly, the culture model we employed proved to be inappropriate since MCF10A cells failed to express the E2 ligase (i.e., UbcH5a) putatively targeted by pAA. Nevertheless, cotreatment with ≥ 50 μM pAA suppressed TCDD-induced AhR degradation in a concentration-dependent manner. Based upon EMSA results, this activity most likely reflects that ability of pAA to function as an AhR antagonist and suppressor of AhR activation at such concentrations.

A second pAA-related activity became apparent at concentrations ranging from 10 and 50 μM. Specifically, treatment of MCF10A cultures with these concentrations prior to TCDD addition elevated CYP1A1 mRNA contents above that occurring with dioxin alone. We previously reported that this concentration range of pAA induces a graded cytostatic response in MCF10A cultures (Elliott and Reiners, 2008). The studies reported in Fig. 6A indicate that 25 μM pAA rapidly initiated simultaneous G1 and S phase arrest in MCF10A cultures. Arrested G1 cells begin to transition into S phase ~7-10 h post pAA treatment, and continued to accumulate in S phase for at least an additional 8 h. Thereafter, S phase cells transitioned into G2. By analyzing the effects of adding TCDD at different times after pAA treatment we were able to assess the responsiveness of CYP1A1 in highly enriched phases of the cell cycle. The studies depicted in Figs. 6B-E indicate that dioxin induction of AhR occupancy of the CYP1A1 promoter and CYP1A1 transcription are optimal in late G1 MCF10A cultures, and as cells transition from G1 into S phase, and during early/mid S phase. This cell cycle phase dependency provides an explanation for the bell shaped kinetics of CYP1A1 mRNA accumulation observed in Fig. 1C. Specifically, the times at which pAA potentiated TCDD-induced CYP1A1 mRNA accumulation
in MCF10A cultures corresponded to the period at which cultures contained the highest percentage of late stage G\textsubscript{1} and S phase cells.

Our observation of a cell cycle dependency for AhR agonist-induction of CYP1A1 is consistent with two other independent studies. Specifically, we previously used centrifugal elutriation of asynchronous cycling U937 cultures to isolate populations in defined stages of the cell cycle (Santani et al., 2001). Subsequent treatment of these populations with TCDD showed a gradient of CYP1A1 mRNA accumulation, with maximal accumulation occurring in late G\textsubscript{1} and early S phase cells. Jiao et al. (2007) used several approaches to generate MCF-7 cultures enriched in G\textsubscript{o}/G\textsubscript{1}, S or G\textsubscript{2}/M phase cells. Maximum accumulation of CYP1A1 mRNA occurred in S phase enriched cultures following short term exposure to the AhR agonist benzo[a]pyrene. In addition to CYP1A1, the ligand-activated AhR regulates the transcription of numerous genes having XREs in their promoters (Sun et al., 2004). In the current study we observed that the induction of CYP2S1 by TCDD in MCF10A cultures was also cell cycle-dependent. Similarly, Jiao et al. (2007) reported maximal accumulations of CYP1A2 and CYP1B1 mRNA in S phase MCF-7 cultures following benzo[a]pyrene treatment. Hence, agonist induction of multiple AhR-responsive genes appears to be cell cycle-dependent in MCF10A and MCF-7 cultures. However, given that similar effects were not observed in HepG2 cultures, it appears that the cell cycle dependency of CYP1A1 induction may be cell type specific. We do not know the basis for this cell context dependency, but it also extends to other aspects of CYP1A1 regulation in the three cell lines. Whereas pretreatment of MCF10A (Guo et al., 2001) and MCF-7 (Moore et al., 1993) cultures with 12\textasciitilde O-tetrade canoylphorbol-13-acetate initially suppresses TCDD-mediated induction of CYP1A1, a similar pretreatment enhances CYP1A1 transcription in HepG2 cultures (Chen and Tukey, 1996; Morgan et al., 1998).
Two timekeepers appear to regulate TCDD-mediated transcriptional activation of *CYP1A1*. The first entails the cell cycle and the second circadian rhythms. Regarding the latter, TCDD-mediated induction of *CYP1A1* in the liver and mammary glands of mice is 23-40 fold greater at night than during daytime (Qu et al., 2007; Qu et al., 2010). This diurnal rhythm in *CYP1A1* responsiveness to TCDD is inversely related to the expression of the protein Period 1 (Metz et al., 2006; Qu et al., 2010), a key protein regulator of the circadian clock (Reppert and Weaver, 2002). The occurrence of this cyclical rhythm for *CYP1A1* responsiveness in a quiescent tissue such as the liver emphasizes the independence of the two timekeepers in some situations. However, several studies have demonstrated that circadian rhythms gate the expression/activation of cell cycle related genes in proliferating tissues (Matsuo et al., 2003; Yang et al., 2009). For example, in normal human oral mucosa the maximum accumulation of Period 1 occurs in G1 prior to the expression of cyclin E, and the onset of S phase occurs after appreciable Period 1 loss (Bjarnason et al., 2001). A similar pattern is also observed in human skin (Bjarnason et al., 2001). Although speculative, it is conceivable that cell cycle and circadian rhythm dependent regulation of TCDD-induced *CYP1A1* transcription maybe linked *in vivo*, in some proliferating tissues.

Protocols that facilitate manipulation of cell density indicate that cell-cell contact also influences AhR activation and the transcription of several AhR-responsive genes (Cho et al., 2004; Ikuta et al., 2004; and references within). Specifically, the expression of both CYP1A1 and CYP1B1 in cultured adherent cells, in the absence of any exogenous AhR ligand, is inversely related to cell density (Cho et al., 2004; Ikuta et al., 2004). In some instances the level of CYP1A1 or CYP1B1 expression induced by culturing at low density or suspension culturing approximate what is achieved following treatment of near confluent, adherent cultures with AhR
agonists (Cho et al., 2004; Ikuta et al., 2004; and references within). Interestingly, studies with cultured keratinocytes (Ikuta et al., 2004) and fibroblasts (Cho et al., 2004) indicate that cell contact/density-mediated regulation of the AhR and CYP1A1/CYP1B1 is cell cycle phase independent.

Agonist activation of the AhR can have diverse effects on the cell cycle. For example, contact inhibited, quiescent rat liver epithelial WB-F344 cultures reenter the cell cycle and proliferate following exposure to TCDD and several polychlorinated biphenyl and polycyclic aromatic hydrocarbon AhR agonists (Chramostová et al., 2004; Vodrácek et al., 2005). Conversely, TCDD induces a profound G1 or S phase arrest in a variety of cultured cell types (reviewed by Marlowe and Puga, 2005; Puga et al., 2009; Barhoover et al., 2010). In many cases this arrest has been shown to be AhR dependent (Marlowe and Puga, 2005). The MCF10A cells used in the current study appear to represent a third case. Specifically, concentrations of dioxin sufficient to induce CYP1A1 transcription had no notable effect on MCF10A cell cycle progression. Collectively, these data indicate that dioxin effects on the cell cycle are cell context dependent. However, it should be noted that even for a specific cell type, additional factors radically influence dioxin’s effects on the cell cycle. For example, whereas dioxin suppresses compensatory liver regeneration induced by partial hepatectomy, it enhances hepatocyte proliferation induced by the hepatomitogen 1,4-bis[2-(3,5-dichloropyridyloxyl] benzene (Mitchell et al., 2010).

In summary, the current study demonstrates that dioxin-mediated transcriptional activation of CYP1A1 is optimal in late G1 and early/mid S phase MCF10A cells. Similar observations have been made in other cell lines (Santani et al., 2001; Jiao et al., 2007). These findings raise the broader issue of whether other genes associated with metabolic transformation
are cell cycle regulated. A recent report by Sugatani et al. (2010) demonstrated that UGT1A1 and CYP2B6 content in HepG2 cells inversely correlated with the activation state of the cell cycle regulated protein cyclin-dependent kinase 2. Knowledge of the cell cycle dependency of drug-metabolizing enzymes may be useful in the design of combinational therapeutic protocols in which one of the therapeutics is a cell cycle modulator.
Authorship Contributions

Participated in research design: Elliott, Joiakim, Kocarek and Reiners, Jr.

Conducted experiments: Elliott, Joiakim, Mathieu, Duniec-Dmuchowski and Reiners, Jr.

Contributed new reagents or analytic tools: Elliott, Joiakim and Kocarek

Performed data analysis: Elliott, Joiakim, Mathieu, Duniec-Dmuchowski and Reiners, Jr.

Wrote or contributed to the writing of the manuscript: Elliott and Reiners, Jr.
References


Guo M, Joiakim A, Dudley DT, and Reiners Jr JJ (2001) Suppression of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated CYP1A1 and CYP1B1 induction by 12-0-


Footnotes

This work was supported in part by the National Institutes of Health Heart, Lung and Blood Institute [Grant HL050710].
Figure legends.

FIG. 1. Effects of pAA on CYP1A1 mRNA accumulation. (A) MCF10A cultures were treated with 1 to 200 μM pAA for 6.5 h prior to harvest, or pretreated with pAA for 1.5 h prior to the addition of 10 nM TCDD, and harvested 5 h later for analyses of CYP1A1 and 7S RNA. (B) Cultures were treated with nothing, 10 nM TCDD, or pretreated with 1 to 100 μM pAA for 1.5 h prior to the addition of TCDD. Cultures were harvested 8 h after the addition of TCDD for analyses of CYP1A1 and 7S RNA. At any specific pAA concentration data are presented for two independent RNA preparations. Data in the lower panel represent means ± SD of 3-5 independent experiments in which RNAs were isolated either 5 or 8 h after TCDD addition. *Different than the CYP1A1/7S ratio obtained with 1 μM pAA, p<0.05. (C) Cultures were treated with 10 nM TCDD or pretreated with 25 μM pAA for 1.5 prior to the addition of TCDD. Cultures were subsequently harvested 3 to 18 h after TCDD addition for analyses of CYP1A1 and 7S RNA. Data in the lower panel represent means ± SD of 3-4 independent experiments in which the CYP1A1/7S ratio for the TCDD only sample, at each time point, was set as one. *Greater than ratio measured at 3 h, p<0.05. (D) Cultures were treated as in panel C but analyzed for CYP1A2 and 7S RNA. Similar results were obtained in a second experiment.

FIG. 2. Effect of pAA on CYP1A1 transcription. (A) Schematic representation of the region of CYP1A1 hnRNA analyzed by real time PCR. (B and C) MCF10A cultures were left untreated, or treated with DMSO, 10 nM TCDD, 25 μM pAA, or 10 nM TCDD + 25 μM pAA for varying lengths of time prior to being harvested and processed for analyses of CYP1A1 hnRNA and GAPDH mRNA. Treatments groups are defined in the figure. Data in B represent means ± SD of
three technical replicates per treatment for a single experiment. Data in C represent means ± SD of three biological replicates for a single experiment. Similar results were obtained in two additional experiments. In co-treatment protocols, pAA was added 1.5 before TCDD. Harvest times are relative to TCDD addition. *Greater than the corresponding TCDD group, $p<0.05$.

FIG. 3. Effect of pAA on CYP1A1 mRNA stability. MCF10A cultures were exposed to 10 nM TCDD or 10 nM TCDD + 25 μM pAA for 8 h to induce CYP1A1 transcription. Actinomycin D (ActD) was then added to 5 μg/ml, without medium change, to inhibit transcription. Cultures were harvested at various times after ActD addition. Treatments are noted in Figure. (A) Northern blot analysis of CYP1A1 and 7S RNAs. One of 4 representative experiments is shown. (B) Semi-log graph of normalized CYP1A1 mRNA contents. mRNA amount is expressed as a percent of treatment at T = 0 (set as 100%). Data represent means ± SD of 4 independent experiments. ANOVA analyses indicated that the curves were not significantly different.

FIG. 4. Effects of pAA on TCDD-induced AhR degradation. (A) MCF10A cultures were treated with 100 μM pAA for 7.5 h prior to harvest, or pretreated with 1 to 100 μM pAA for 1.5 h prior to the addition of 10 nM TCDD. Cultures were harvested 6 h after TCDD addition for analyses of AhR and actin content by western blot. Similar results were obtained in two additional experiments. (B) Cultures were treated with nothing, 10 nM TCDD, or pretreated with 50 μM pAA for 1.5 h prior to the addition of TCDD. Cultures were harvested 1-6 h after the addition of TCDD for analyses of AhR and actin proteins. (C) Cultures were treated with 10 nM TCDD, DMSO or 25 μM pAA for 1.5 h prior to TCDD addition, and subsequently harvested 2-10 h after TCDD addition for analyses of AhR and GAPDH by western blot. The lower panel represents
means ± SD of 4 independent experiments employing 25 μM pAA pretreatment. Relative quantification was achieved by first calculating AhR/GAPDH ratios, and then normalizing to the ratio of non-treated cultures, which was set as 100%. The AhR contents of the two treatment groups were not statistically different from one another at any of the time points. Western blots used 25 μg of protein lysate.

FIG. 5. AhR antagonist properties of pAA. (A) Rat liver extract was incubated with 10 nM TCDD or different concentrations of pAA prior to the addition of a radiolabeled oligo containing a consensus XRE, and subsequent EMSA. (B) Rat liver extract was coincubated with 10 nM TCDD and 1 to 200 μM pAA prior to the addition of a radiolabeled oligo containing a consensus XRE, and subsequent EMSA. Parallel reaction mixtures containing a 50-fold excess of unlabeled oligo were used to control for non-specific AhR-DNA interactions. Similar results were obtained in a second independent study.

FIG. 6. TCDD-induced CYP1A1 transcription in MCF10A cultures is cell cycle dependent. (A) MCF10A cultures were exposed to 25 μM pAA and subsequently harvested 1.5 to 21 h later for analyses of the percentages of cells in G1, S and G2/M. (B) Cultures were treated with 25 μM pAA and at varied times thereafter exposed to 10 nM TCDD for an additional 3 h prior to processing for cell cycle analyses. Each column in panels A and B represent analyses of 2x10^4 cells. (C) Cultures were treated as in panel B and harvested for northern blot analyses of CYP1A1 and 7S RNAs. The lower panel represents quantification of the northern blot data. (D) Real time RT-PCR analysis of CYP1A1 hnRNA in the RNA preparations used in panel C. The data presented in panels A-D are from a single experiment. A second experiment that
encompassed the same types of analyses as presented in panels B and C yielded very similar results. (E) MCF10A cultures were exposed to 25 μM pAA for 1.5, 11 or 18 h prior to the addition of 10 nM TCDD. Cultures were subsequently harvested 0.5, 2 or 3 h after dioxin addition for ChIP analyses of AhR occupancy of the CYP1A1 promoter.

FIG. 7. TCDD-induced CYP2S1 transcription in MCF10A cultures is cell cycle dependent. (A) MCF10A cultures were left untreated or pretreated with 25 μM pAA for different lengths of time prior to the addition of 10 nM TCDD. At parallel times some untreated cultures were treated with DMSO or 10 nM TCDD. All cultures were harvested 3 h after the additions of either DMSO or TCDD for isolation of RNAs and real time PCR analyses of CYP2S1 mRNA and 18S ribosomal RNA. Relative CYP1S1 content was determined as described in Materials and Methods using the no treatment group value set as 1. Data represent means ± SD of analyses involving three independent culture dishes per time point and treatment group. *Greater than no treatment, DMSO-treated, and TCDD-only treated groups, p<0.05. (B) MCF10A cultures were exposed to 25 μM pAA and subsequently harvested 1.5 to 21 h later for analyses of the percentages of cells in G1, S and G2/M. Each column represents analyses of 2x10⁴ cells.

FIG. 8. Effects of pAA on CYP1A1 mRNA accumulation in HepG2 cultures. (A) HepG2 cultures were treated with different pAA concentrations, for different lengths of time, before being harvested for assessment of cell number (closed symbols) or viability (open symbols; absence of trypan blue staining). Data represent means ± SD of analyses performed on three independent plates per time and treatment group. Error bars are hidden in many cases by symbols. Treatments are noted in the figure. (B) HepG2 cultures were exposed to 25 μM pAA and subsequently
harvested 8 to 28 h later for analyses of the percentages of cells in G₁, S and G₂/M. Each column represents analyses of 2×10⁴ cells. (C) HepG2 cultures were treated as described in Fig. 7A, and processed for real time PCR analyses of CYP1A1 and 18S ribosomal RNA. Data represent means ± SD of analyses involving three independent culture dishes per time point and treatment group. *Greater than no treatment, DMSO-treated, and TCDD-treated groups, p<0.05.
Figure 1

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Relative 1A1/7S

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Relative 1A1/7S

0.83 0.81 1.2 1.9 2.3 2.0 2.2 2.8 0.99 0.83 0.44

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Relative 1A1/7S

1.0 1.84 7.51 1.32 1.53 1.14 2.32 3.88 3.17 3.14

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Relative 1A2/7S

1.0 0.58 0.19 1.09 1.44 1.84 2.61 2.60 2.18

Relative 1A2/7S

1.0 0.56 0.19 1.09 1.44 1.84 2.61 2.60 2.18

* Significant difference from control
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#### TCDD + pAA

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### B

**% CYP1A1 Remaining**

- **TCDD**: 4.9 ± 0.8
- **TCDD + pAA**: 4.0 ± 0.8

*Graph shows the decay of CYP1A1 mRNA half-life over time after ActD addition.*
Figure 4

A TCDD — — + + + + + + + +
pAA (μM) — 100 100 75 50 25 10 1
AhR
Actin
Relative AhR/Actin
1.0 0.82 0.67 0.46 0.36 0.19 0.18

B pAA (50 μM) — — — — — — + + + +
TCDD — + + + + + + + + + + + +
AhR
Actin
Relative AhR/Actin
1.0 0.32 0.23 0.20 0.51 0.46 0.70

C Time (h) 0 2 4 6 8 10 2 4 6 8 10
pAA (25 μM) — — — — — — + + + + +
DMSO — + + + + + + — — — —
AhR
GAPDH
Relative AhR/GAPDH
1.0 0.36 0.16 0.12 0.12 0.18 0.31 0.28 0.29 0.39

% AhR Remaining

Time (h)

0 5 10 15

[Graph showing % AhR Remaining over time with data points for TCDD and TCDD + pAA]
Figure 5

A  Cold Oligo  - - - - - - - +  B  - - - - - - - +
   TCDD  - + - - - - - -  +  - + + + + + + + +
       pAA (μM)  - - 1 10 50 100 200 -  - - 1 10 50 100 200 -

AhR-oligo

Free Oligo

Relative AhR-oligo complex content  1.0  0.82  0.84  0.53  0.34  0.30
Figure 6

A. Distribution of cells across different phases of the cell cycle (% of total cells) over time after pAA addition (h).

B. Distribution of cells across different phases of the cell cycle (% of total cells) over time after TCDD addition after pAA addition (h) and time of harvest (h).

C. Time of harvest (h) and TCDD addition after pAA (h) for CYP1A1 and 7S expression.

D. Graph showing fold change in CYP1A1 mRNA over time after TCDD addition after pAA (h) and time of harvest (h).

E. Table showing time after pAA addition (h), time after TCDD (h), and AhR/Input ratio.
Figure 7

A

Relative CYP2S1 mRNA Content

- X: No Treatment
- O: DMSO
- △: TCDD
- ▲: TCDD + pAA

TCDD addition after pAA (h)

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Time of harvest (h)

B

% of Total Cells

- □: G1
- □: S
- □: G2/M

Time after pAA addition (h)

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**Supplemental Figure 1.** Absence of TCDD effect on pAA induced G1 and S phase arrest. Cultures of MCF10A cells were treated with nothing, DMSO alone, 25 µM pAA alone, 10 nM TCDD alone, or with pAA for 1.5 h prior to the addition of TCDD. Cultures were harvested at various times after TCDD addition for FACS analyses of cell cycle distribution. Data represent analyses of 20,000 cells. Similar results were obtained in two additional experiments.